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Use of fecal samples for microbiome analysis in bumblebees

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Abstract

Use of fecal samples for microbiome analysis in bumblebees

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Due to declines in bumblebees and other pollinators, there is an increased need for monitoring of bee populations and health. The gut microbiome is integral to bumblebee health, with roles in nutrition and immune function, including interactions with pathogens, which have been shown to contribute to bee declines. Noninvasive methods enable deeper sampling of bee populations with less impact on sensitive populations. Wider sampling of bumblebee microbiomes would provide information on bee health while expanding the phylogenetic and ecological scope of bee microbiome research. Previous studies have demonstrated the use of fecal samples to obtain bee DNA, and that fecal microbiomes are able to recover gut microbiomes. This study demonstrates the use of fecal samples for comparative microbiome analyses using two bumblebee species from North America.

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INTRODUCTION

Declines of bumblebee populations have been reported in North America (Cameron et al 2010) and Europe (Goulson et al 2008), impacting both agriculture and wild ecosystems. Some species continue to thrive across their historical ranges, while others, such as *Bombus affinis*, are now endangered (IUCN 2018). Due to the sensitivity of these populations, nonlethal and noninvasive methods are of increasing necessity for bumblebee monitoring. Existing nonlethal DNA sampling methods in bees include tarsal segments (Holehouse et al 2003) and wing clippings (Chaline et al 2004). Noninvasive methods that leave the sampled individual fully intact include photographic vouchers for morphological assessments (Thomson and Zung 2015) and fecal samples for DNA (Scriven et al 2012).

In recent years, there is a growing understanding of the role of host-associated microbes in insect life history and ecology. In corbiculate bees, the gut microbiome has coevolved with the major lineages, and studies in honeybees have shown the microbiome to be critical to bee nutrition through fermentation of carbohydrates (Lee et al 2014), digestion of sugars otherwise toxic to the host (Zheng et al 2016), and regulation of appetite (Zheng et al 2017). In addition to nutritional function, the bee gut microbiome is a major player in interactions with pathogens, of particular relevance in the context of bee population declines. In South America, declines in native species have been attributed to pathogens among other factors (Schmid-Hempel et al 2013, Arbetman et al 2013), and declining species in North America have higher prevalence of the microsporidian pathogen *Nosema bombi* (Cameron et al 2011). In bumblebee infections by the trypanosome *Crithidia bombi*, differences in gut microbiome composition lead to differences in infection intensity (Mockler et al 2018), and bees devoid of gut bacteria suffer higher infection loads (Napflin and Schmid-Hempel 2018). In honeybees, the gut microbiome has been shown to prime the innate immune system, impacting generalized responses against a wide range of enemies (Schwarz et al 2015, Kwong et al 2017). Because the gut microbiome is critical to bee health, wider sampling of this aspect of bee life history may provide insights into the dynamics of bumblebee population resilience.

In mammals and other organisms where a direct gut sample is difficult to obtain, gut microbiomes are commonly studied using fecal samples. For most wild mammals, the study of resident microbiomes relies on feces (Schmidt et al 2018). Feces have been used as a proxy for the gut microbiome in other insects, including in Lepidoptera where the transient gut microbiome is diet-derived and not host-adapted (Hammer et al 2017), and in German cockroaches, where there is high correlation between gut and fecal microbiomes (Kakumanu et al 2018). Much of the work on bee gut microbiomes to date has been done on honeybees and a few other bee species that can be reared reliably. Collecting the gut microbiome requires sacrificing the bee, and this may not be viable for some rare or elusive species, or in threatened populations. Unlike honeybees, bumblebees will defecate in an enclosed space, allowing sampling of the gut microbiome without harming the bee. The use of feces to collect the gut microbiome has the potential to expand the range of species included in microbiome studies, and may enable monitoring of microbiome health without destructive sampling.

A previous study (Napflin and Schmid-Hempel 2018) has demonstrated that bumblebee feces contain similar taxa as the gut microbiome. In this study, we expand this use of feces for monitoring the bumblebee gut microbiome to a species comparison of two North American species using lab-reared *Bombus impatiens* and *Bombus pensylvanicus*. *B. impatiens* is still widespread across its historical range, and is reared for agricultural use. *B. pensylvanicus* has declined in most of its range, except for Texas. This is the first example of *B. pensylvanicus* reared in captivity, and the shared lab environment presents an opportunity to compare host effects on the microbiome while keeping environmental factors such as temperature and diet constant.

METHODS

Sample collection

All bees in this study were workers from colonies reared from wild mated queens. *Bombus impatiens* and *Bombus pensylvanicus* queens were collected in San Jacinto County, Texas (30.7410036, -953186187) in March 2015, and encouraged to start a colony following previously described methods (Mockler et al 2018). Colonies were fed sterile irradiated pollen (Betterbee, Greenwich, NY) and sucrose water *ad libitum* and provided with a chamber attached to the main colony container for waste disposal.

Feces and gut samples were obtained from 8 *B. impatiens* and 9 *B. pensylvanicus* workers. Feces were collected by placing the bee in a sterilized plastic vial and allowing it to defecate, agitating the vial to encourage a response if necessary. The bee was then transferred to another holding vial while the feces were retrieved with a micropipette. Feces volumes and consistency varied among individuals and were not recorded, ranging between roughly 3ul to 15ul. After collection of feces, the bee was anesthetized by chilling, and the whole gut was dissected for DNA extraction. Feces and gut samples were homogenized in 20% glycerol and stored at -80C for one month prior to DNA extraction. DNA extraction and PCR amplification of the V4 region of the bacterial 16S rRNA gene were performed as previously described (Raymann et al 2017). 34 samples with the highest concentration of genomic DNA were retained for sequencing. Amplicons were sequenced using the Illumina MiSeq platform (2x250bp) at the University of Texas Genomic Sequencing and Analysis Facility.

Microbiome analysis

Sequences were processed in Qiime2 version 2018.8 (Hall and Beiko 2018). Reads were demultiplexed using the plugin demux (https://github.com/qiime2/q2-demux). Quality control, denoising, and amplicon sequence variants (ASV) calling were done using DADA2 through the plugin q2-dada2 (Callahan et al 2016). After quality control, 32 samples remained in the analysis. ASVs were assigned taxonomy using a naïve Bayes classifier pre-trained on the SILVA database 132 release with 99% OTUs from the 515F/806R region of 16S. Chloroplast sequences were filtered out, resulting in 44 ASVs remaining in the analysis. Diversity metrics were

calculated using the core-metrics function. Taxon abundances were analyzed as relative abundance instead of absolute abundance, because fecal samples varied in volume and density, rendering absolute counts uninformative for comparison between samples. Diversity metrics were conducted with default parameters, on reads rarefied to a sampling depth of 260 reads per sample, which maximized both read depth and sample retention.

Statistical analysis

Differences between sample groups in alpha diversity metrics was analyzed using the Kruskal-Wallis H test. Beta diversity was characterized with the Bray-Curtis dissimilarity index, and differences tested using PERMANOVA. Gut and feces beta diversity ordinations were compared using a Mantel test. Differences in taxonomic composition between sample groups was analyzed using ANCOM. All analyses were conducted in Qiime2.

RESULTS

Taxonomic assignments of OTUs indicated that the major groups of bee bacteria were present in these samples (Table 1, Fig. 1). *Gilliamella* and *Snodgrassella* were present in all samples, and Lactobacillus Firm-5 and bee-associated *Bifidobacterium* were present in most samples. Additionally, samples contained occasional plant-associated bacteria. *B. impatiens* and *B. pensylvanicus* differ in gut alpha diversity. *B. impatiens* had greater gut microbiome OTU richness than *B. pensylvanicus* (Kruskal-Wallis test H= 10.9178, p= 0.00095. And B. impatiens gut had lower Pielou's Evenness than pensylvanicus (H= 6.48214, p= 0.010896) (Fig 2).

The microbiomes of *B. impatiens* and *B. pensylvanicus* differed in composition, as indicated by samples clustering separately in the ordination of Bray-Curtis dissimilarity index (PERMANOVA 999 permutations gut samples: F=11.2743, p=0.001, feces samples: F=5.1437, p=0.003)(Fig 3). These differences between species were evident in both gut and feces samples indicating that feces may reflect species differences in gut microbiota. However, although the separation between *B. impatiens* and *B. pensylvanicus* gut community composition was recapitulated in feces, a given individual bee's feces community was not able to predict the same bee's gut community composition among other individuals. The ordination of feces community dissimilarity did not correlate with the ordination of gut communities from the same individuals (Mantel test on Bray-Curtis dissimilarity, 999 permutations, Spearman rho=0.17408, p=0.053).

Fecal OTU richness did not differ significantly from gut richness (*B. impatiens* H=3.221, p=0.0727. *B. pensylvanicus* H=1.803, p=0.179) (Fig 4). Feces and gut communities differed in evenness within species, but the direction of change was not consistent, indicating a lack of a systematic difference between feces and gut. In both bee species, no bacterial genera differed significantly in abundance between feces and gut samples (Qiime2 plugin ancom).

DISCUSSION

Noninvasive sampling of bee gut microbiomes has the potential to be a valuable tool for bumblebee monitoring, and can expand the range of bee species included in microbiome studies. This study tests the use of feces in a comparison of gut microbiomes of two *Bombus* species.

Gut and feces microbiomes were dominated by the five core bacterial lineages known to have evolved with *Bombus* and other corbiculate bees, and OTU richness in both were similar in magnitude to other wild-collected bumblebee species (Kwong et al 2017). The Gram-negative clusters (*Gilliamella* and *Snodgrassella*) were present in all samples, and the Gram-positive clusters (*Lactobacillus*, Firm-5 and *Bifidobacterium*) were present in most. Previous work has shown that strains within the major clusters differ among *Bombus* species, with some strains being species specific and others being generalists (Powell et al 2016). Although we did not examine strain level differences, which require use of more variable genetic markers, the presence of the major bee-associated bacterial taxa in feces from *B. impatiens* and *B. pensylvanicus* implies that such variation could be surveyed using fecal samples. Further work on bee microbe strain diversity can be conducted using only fecal samples without the need for destructively sampling the bee, opening up the study of core bee bacteria to wider host sampling. Expanding the range of hosts studied for strain diversity would clarify the patterns of bacterial strains' adaptation to the host and host switches.

Previous studies have shown that sympatric *Bombus* species differ in microbiome composition (Cariveau et al 2014). In this study, *B. impatiens* and *B. pensylvanicus* gut microbiomes also differed in gut microbiome composition, despite having been reared under the same conditions. The two *Bombus* species differed in whether feces or gut has higher diversity, likely resulting from small sample size and stochastic differences between individuals and defecation events. In *B. pensylvanicus, Snodgrassella* and *Gilliamella* comprised a greater proportion of the fecal community than the gut community, while this contrast did not exist in *B. impatiens*. This may reflect differences between *B. impatiens* and *B. pensylvanicus* in the spatial organization of bacterial taxa within the gut, or differences in the mechanics of defecation. Community composition was significantly different between bee species in both gut and feces, and there

were no systematic differences in diversity between the gut microbiome and the feces microbiome. However, while gut community differences between bee species were recapitulated in feces communities, fecal samples were not strong predictors of individual variation in gut community composition.

In the wild, differences among bee species in foraging behavior and diet choice may contribute to some differences in the core bacterial taxa. Additionally, wild bee workers would be expected to exhibit greater variation in their microbiomes due to seasonal changes (Ludvigsen et al 2015), degradation of their symbiotic microbiota through aging (Hroncova et al 2015) or exposure to xenobiotics (Motta et al 2018) and contact with environmental bacteria (Parmentier et al 2015). Due to this greater variation, feces may be more effective at capturing individual microbiome idiosyncrasies in wild bees compared to lab-reared bees.

In vertebrates, fecal samples are standard as a proxy for the gut (Schmidt et al 2018) despite the potential for systematic differences between the two sample types (Tang et al 2015) that are often not explicitly characterized prior to using fecal samples. Bumblebee feces have been shown to retrieve a sample of the gut microbiome, and can be used for studies of community composition. The use of fecal samples would expand the scope of bee species and populations for which microbiome studies are possible. Because of the low impact on sampled populations, fecal sampling could contribute to high-throughput ecological monitoring, and adding microbiome characteristics to monitoring of threatened and rare bees. Noninvasive sampling could also expand the phylogenetic scope of bees included in studies of broader evolutionary patterns of the bee gut microbiome.

FIGURES

	B. impatiens		***B. pensylvanicus***	
	Gut n=8	Feces n=8	Gut n=7	Feces n=9
Gilliamella	100%	100%	100%	100%
Snodgrassella	100%	100%	100%	100%
Lactobacillus	100%	100%	86%	100%
Bifidobacteria	88%	88%	57%	67%
Bartonella	25%	25%	43%	0%

Table 1. Percentages of samples that contain each bacterial lineage.
Based on OTU assignments from QIIME2, the major groups of bee-associated bacteria are present in most sampled individuals. Recovery of bacterial groups from each individual bee does not differ greatly between feces and gut as microbiome sources.

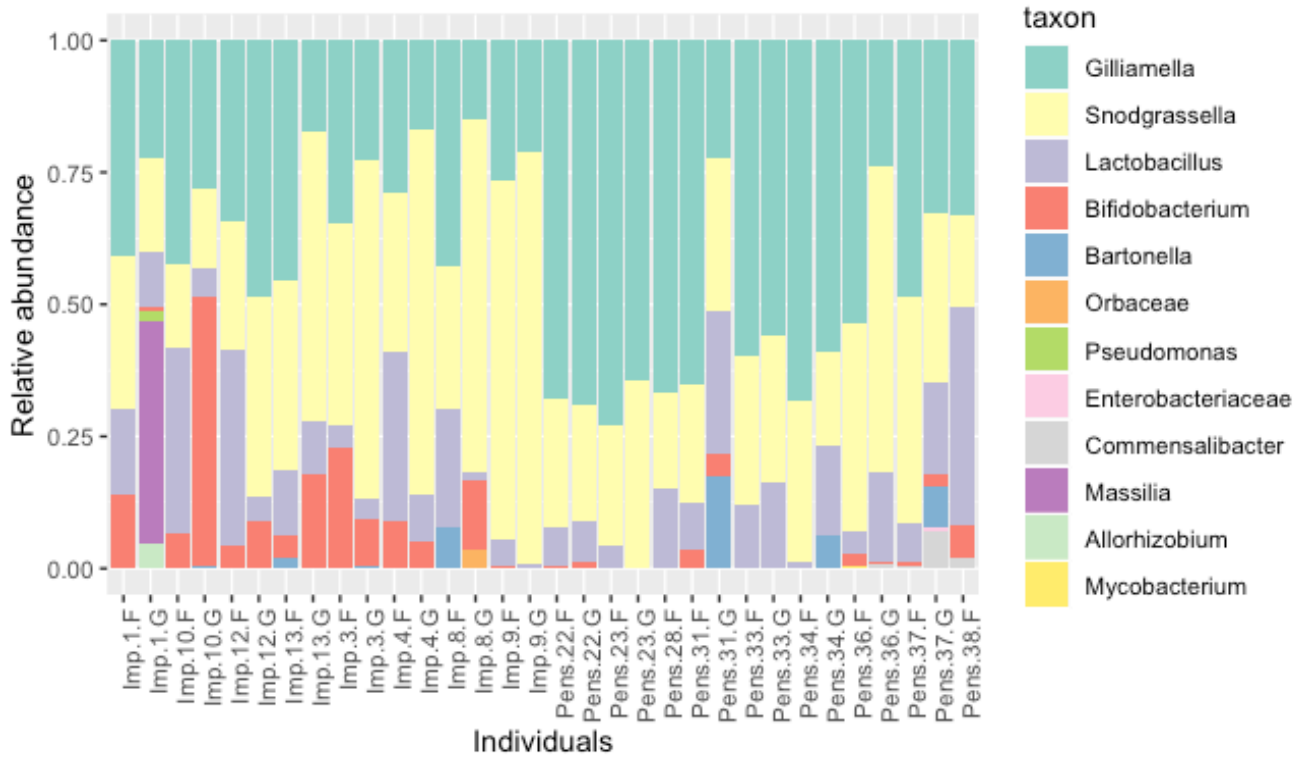

Fig 1: Composition of bacterial communities retrieved from feces and guts of bumble bees. Relative abundances of bacterial taxa are shown at QIIME2 taxonomic level 6. X axis labels indicate bee species (Imp = *B. impatiens,* Pens = *B. pensylvanicus*), individual number (1-38), and sample type (F=feces, G=gut). Each bar represents a single microbiome sample (feces or gut), grouped by bee individual. Bacterial relative abundances vary between bee individuals, and between feces and gut from the same individual. Although most major taxa of bee-associated bacteria are present in most samples, *B. impatiens* and *B. pensylvanicus* samples differ in relative abundances of bacterial taxa.

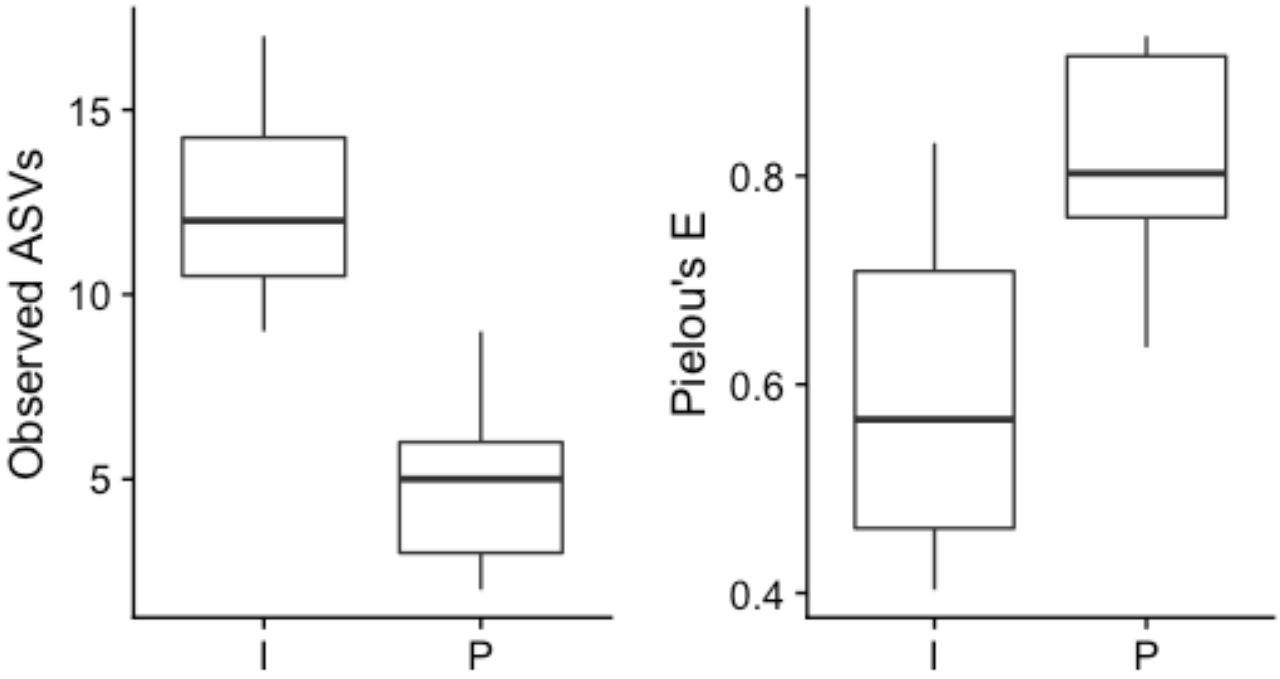

Fig 2. Species richness and evenness in bacterial communities of bumble bee guts. *B. impatiens* gut microbiomes have significantly higher bacterial species richness than *B. pensylvanicus* gut microbiomes (Observed ASVs: Kruskal-Wallis chi-squared = 10.918, df = 1, p-value = 0.001). In *B. impatiens,* bacterial taxa have less even abundance than in *B. pensylvanicus* (Pielou's Evenness Kruskal-Wallis chi-squared = 6.4821, df = 1, p-value = 0.011)

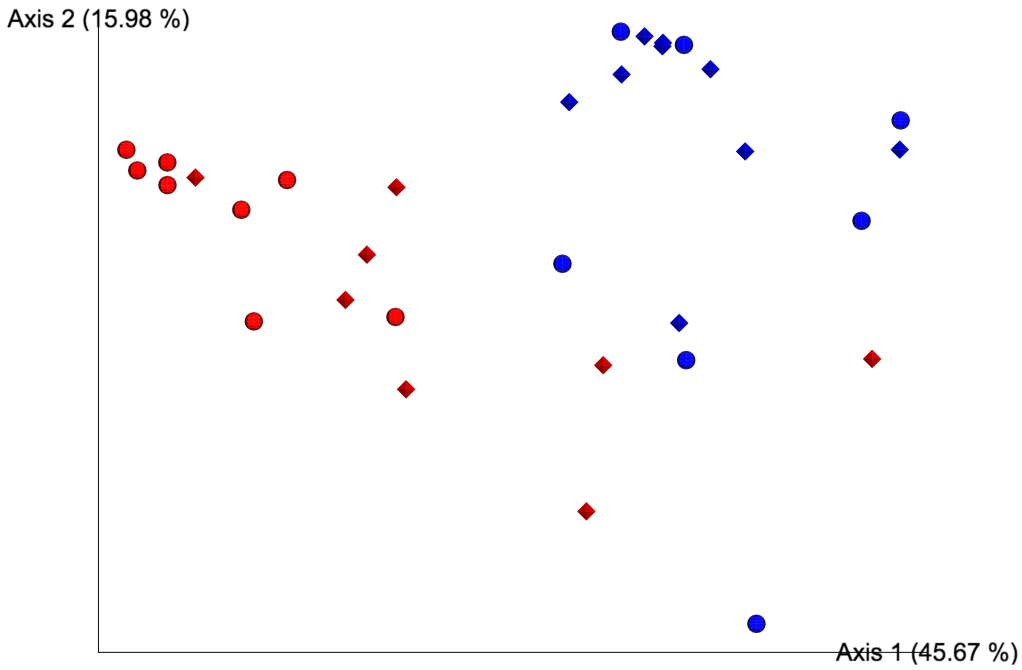

Fig 3: Bray-Curtis dissimilarity
Beta diversity of samples is shown using a principal coordinates analysis (PCoA) plot of Bray-Curtis distances measuring community dissimilarity between samples. Samples that are closer together in the ordination have more similar communities. Variation along Axis 1 accounts for 45.67% of the total variation among sampled communities. Axis 2 accounts for 15.98% of the total variation among sampled communities.
Samples differ in composition between *B. impatiens* (red) and *B. pensylvanicus* (blue). This species difference is present in both gut samples (circles) and feces samples (diamonds).
Gut samples differ in composition between species (n=15, PERMANOVA F=11.2743, p=0.001, 999 permutations). Feces differ in composition between species (n=17, PERMANOVA F=5.1437, p=0.003, 999 permutations). The beta diversity ordinations for gut and feces are not correlated (Mantel test, Spearman rho=0.17408 p-value = 0.053, 999 permutations), indicating that feces and gut communities from the same bee individual are not most similar to each other in composition.

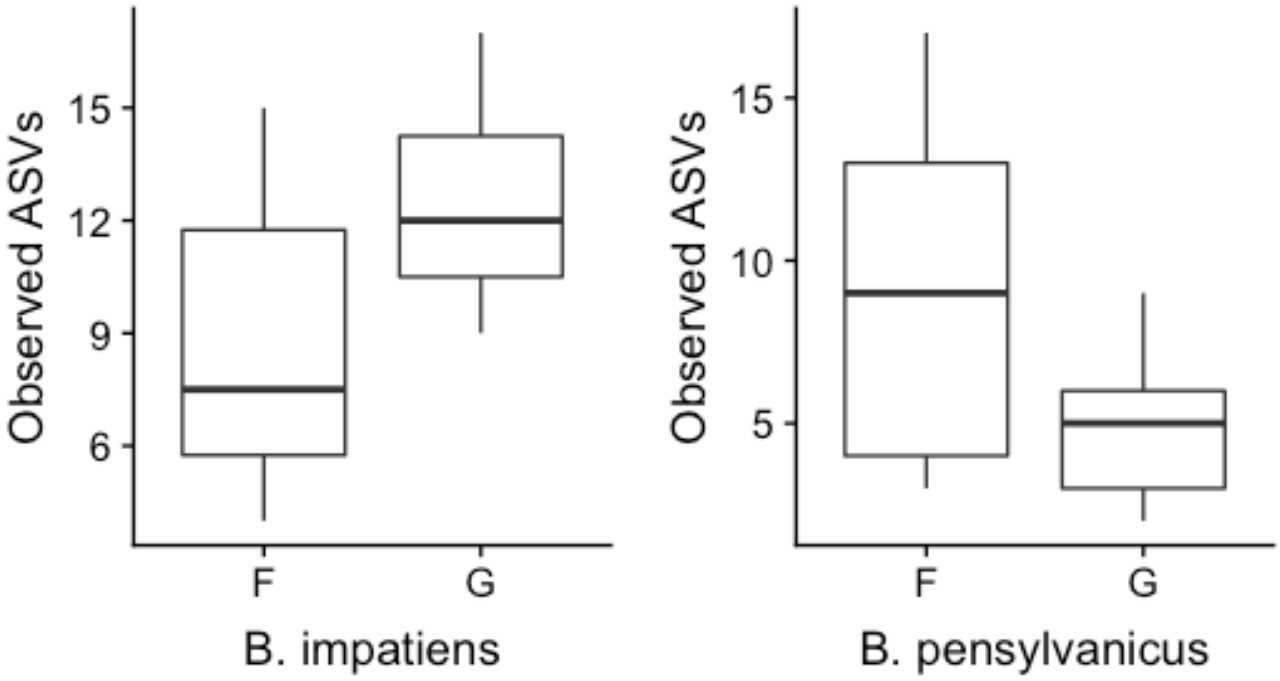

Fig 4. Feces and gut samples do not differ significantly in bacterial species richness in *B. impatiens* (Kruskal-Wallis chi-squared = 3.2207, df = 1, p-value = 0.07271) and in *B. pensylvanicus* (Kruskal-Wallis chi-squared = 1.8028, df = 1, p-value = 0.1794).

References

Arbetman MP, Meeus I, Morales CL, Aizen MA & Smagghe G (2013) Alien parasite hitchhikes to Patagonia on invasive bumblebee. Biological Invasions 15: 489–494

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ & Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13:581-583

Cameron SA, Lozier JD, Strange JP, Koch JB, Cordes N, Solter LF & Griswold TL (2010) Patterns of widespread decline in North American bumble bees. Proceedings of the National Academy of Sciences USA, 108:662-667

Cariveau DP, Powell JE, Koch H, Winfree R & Moran NA (2014) Variation in gut microbial communities and its association with pathogen infection in wild bumble bees. ISME Journal 8:2369-2379

Chaline N, Ratnieks FLW, Raine NE, Badcock NS & Burke T (2004) Non-lethal sampling of honey bee, Apis mellifera, DNA using wing tips. Apidologie 35:311-318

Goulson D, Lye GC & Darvill B (2008) Decline and conservation of bumble bees. Annual Review of Entomology 53: 191–208

Hall M & Beiko RG (2018) 16S rRNA gene analysis with QIIME2. Microbiome Analysis. Pp 113-129.

Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP & Fierer N (2017) Caterpillars lack a resident gut microbiome. Proceedings of the National Academy of Sciences USA 114:9641-9646

Holehouse KA, Hammond RL & Bourke AFG (2003) Non-lethal sampling of DNA from bumble bees for conservation genetics. Insectes Sociaux 50:277-285

Hroncova Z, Havlik J, Killer J, Doskocil I, Tyl J, Kamler M, Titera D, Hakl J, Mrazek J, Bunescova V & Rada V (2015) Variation in honey bee gut microbial diversity affected by ontogenetic stage, age and geographic location. PLoS ONE 10:e0118707

IUCN 2018. The IUCN Red List of Threatened Species. Version 2018-2. <http://www.iucnredlist.org> http://www.iucnredlist.org. Downloaded on 09 October 2018

Kakumanu ML, Maritz JM, Carlton JM & Schal C (2018) Overlapping community compositions of gut and fecal microbiomes in lab-reared and field-collected German cockroaches. Applied and Environmental Microbiology 84:e10137-18

Kwong WK, Mancenido AL & Moran NA (2017) Immune system stimulation by the native gut microbiota of honey bees. Royal Society Open Science 4:170003

Kwong WK, Medina LA, Koch H, Sing KW, Soh EJY, Ascher JS, Jaffe R & Moran NA (2017) Dynamic microbiome evolution in social bees. Science Advances 3:e1600513

Lee FJ, Rusch DB, Stewart FJ, Mattila HR & Newton IL (2014) Saccharide breakdown and fermentation by the honey bee gut microbiome. Environmental Microbiology 17:796–815

Ludvigsen J, Rangberg A, Avershina E, Sekelja M, Kreibich C, Amdam G & Rudi K (2015). Shifts in the midgut/pyloric microbiota composition within a honey bee apiary throughout a season. Microbes and Environments 30:235–244

Mockler BK, Kwong WK, Moran NA & Koch H (2018) Microbiome structure influences infection by the parasite Crithidia bombi in bumble bees. Applied and Environmental Microbiology 84:e02335-17

Motta EVS, Raymann K & Moran NA (2018) Glyphosate perturbs the gut microbiota of honey bees. Proceedings of the National Academy of Sciences USA 115:10305-10310

Napflin K & Schmid-Hempel P (2018) High gut microbiota diversity provides lower resistance against infection by an intestinal parasite in Bumblebees. The American Naturalist 192:131-141

Parmentier L, Meeus I, Mosallanejad H, de Graaf DC & Smagghe G (2015) Plasticity in the gut microbial community and uptake of Enterobacteriaceae(Gammaproteobacteria) in Bombus terrestris bumblebees' nests when reared indoors and moved to an outdoor environment. Apidologie 47:237–250

Powell JE, Ratnayeke N & Moran NA (2016) Strain diversity and host specificity in a specialized gut symbiont of honeybees and bumblebees. Molecular Ecology 25:4461-4471

Raymann K, Shaffer A & Moran NA (2017) Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. Plos Biology 15:e2001861

Schmid-Hempel R, Eckhardt M, Goulson D, Heinzmann D, Lange C, Plischuk S, Escudero LR, Salathe R, Scriven JJ & Schmid-Hempel P (2013) The invasion of southern South America by imported bumblebees and associated parasites. Journal of Animal Ecology 83:823–837

Schmidt TSB, Raes J & Bork P (2018) The human gut microbiome: from association to modulation. Cell 172:1198-1215

Schwarz RS, Huang Q & Evans JD (2015) Hologenome theory and the honey bee pathosphere. Current Opinion in Insect Science 10:1–7

Scriven JJ, Woodall LC & Goulson D (2012) Nondestructive DNA sampling from bumblebee faeces. Molecular Ecology Resources 13:225-229

Tamic T, Odoux JF, Loublier Y, Chabirand M, Aupinel P (2016) Toward a new rapid method to assess plant species in a bee pollen diet. Journal of Apicultural Research 55:107-112

Tang MS, Poles J, Leung JM, Wolff MJ, Davenport M, Lee SC, Lim YA, Chua KH, Loke P & Cho I (2015). Inferred metagenomic comparison of mucosal and fecal microbiota from individuals undergoing routine screening colonoscopy reveals similar differences observed during active inflammation. Gut Microbes 6:48–56

Thomson JD & Zung JL (2015) A restraining device to aid identification of bees by digital photography. Journal of pollination Ecology 17:145-147

Zheng H, Nishida A, Kwong WK, Koch H, Engel P, Steele MI & Moran NA (2016) Metabolism of toxic sugars by strains of the bee gut symbiont Gilliamella apicola. mBio 7:e01326-16

Zheng H, Powell JE, Steele MI, C Dietrich & NA Moran (2017) Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. PNAS. 114:4775-4780